ELISA SECONDARY ANTIBODY CONJUGATION

(Wright *et al.*, 1987 & Wright *et al*, 1996)

Introduction

This assay allows you to determine the concentration of glomalin, specifically. It uses a secondary antibody conjugation and a biotinylated antibody with a long spacer arm for high specificity. This procedure also allows you to use a very tight curve and get very exact concentration values. It is important to note that Dynex u-shaped microtiter plates should be used (see inventory for ordering info). The shape of the well and type of plastic makes a difference in the specificity.

Materials

Dynex 96-well microtiter plate
tilt table or shaker
plate reader with a 405 or 410 filter
2% non-fat milk (2g powdered milk/100ml of PBS)
PBS (Phosphate buffered saline), pH 7.4
PBST (PBS with 0.2 ml/L Tween 20), pH 7.4
1% BSA (1 g BSA/100 ml PBS)*
MAb32B11 antibody (Dilute 1 ml antibody in 5 ml PBS)**
Biotinylated anti-mouse IgM (1:2500 in 1% BSA) (4.8µl/6ml)**
ExtrAvidin peroxidase (1:2000 in 1% BSA) (3µl/6ml)**
color developer (7.5 ml/plate)

*Make about 500-1000 ml of stock and dispense in 6 ml aliquots that will be frozen until needed.
**These are recommended concentrations. Chemicals obtained from different companies or with different lot numbers may need to be optimized for your conditions. If you are not getting the expected results, concentrations may need to be adjusted (see below).

Methods

1) Prepare standard curve.

A. Make up a stock solution of 0.08 ug of protein/100 ul of PBS and store at 4^{o}C (Do not freeze the ELISA stock solution or use it if it appears to be contaminated or is several months old.)

Stock solution:

a) Use protein extracted from fresh hyphae or soil that is nearly 100% immunoreactive. To determine this, run a Bradford and an ELISA assay on the samples and compare concentrations.
b) If Bradford and ELISA values are nearly the same, make an ELISA curve and test the values by comparing results to a known curve.
c) Make up 500 ul aliquots of the stock with a concentration of 0.08 ug of protein in 100 ul of PBS or 0.40 ug of protein in 500 ul of PBS.

B. Put 100 ul stock (concentration in stock = 0.08 ug protein/100 ul of PBS) in 2 of the wells and 50 ul PBS in the other 10 wells.
C. Transfer 50 ul of the 0.08 ug sample to a neighboring well that has 50 ul PBS (Fig. 1).

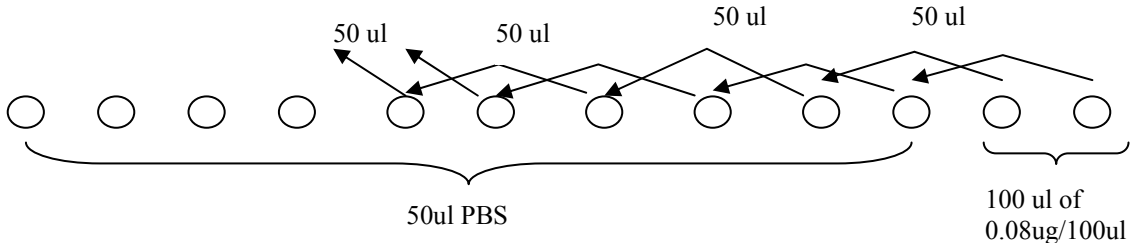

Figure 1. Serial dilution starting with 100 ul of standard solution containing 0.08 ug/100 ul.

D. Mix 3-4 times with the micropipette by pulling the sample up and down.
E. Remove 50 ul from these 2 wells and transfer to 2 neighboring wells (Fig. 1). Mix 3-4x. Repeat for these 2 wells (Fig. 1).
F. After the 3rd dilution, remove 50 ul from the 2 wells that have 100 ul and dispose of it (Fig. 1). The row will then have a standard curve with the values shown in Table 1.

Table 1. Glomalin concentrations in wells.

Well #	Protein concentration (ug/50 ul)
1	0
2	0
3	0
4	0
5	0.005
6	0.005
7	0.01
8	0.01
9	0.02
10	0.02
11	0.04
12	0.04

2) Add samples to wells with volumes calculated from the total protein assay values (as discussed below) plus enough PBS to equal a total of 50 ul or add 50 ul of pre-diluted sample to a well.

Volume of sample needed for each well

Take 0.02 ug (which is a value in the middle of the ELISA curve) and divide by the ug/ul value from the total protein assay. This gives you the volume (ul) needed to give a concentration of 0.02 ug/well. By targeting a concentration of 0.02 ug/well, you can dilute a little more or a little less per sample so you can standardize your dilutions. In other words, volumes for three different samples may be 2.16, 3.04 and 2.78 ul, respectively. Instead of using these three different volumes, just use 2.5 ul for each.

Example:
Total protein assay ug/well concentration = 2.54 ug/well
Total protein assay ul of sample/well = 10 ul/well

0.02 ug ÷ (2.54 ug/well ÷ 10 ul/well) = 0.08 ul ≈ 0.1 ul

- OR -

Pre-dilute the sample by multiplying the volume needed per well by 20 (since there are twenty 50 ul increments in 1 ml). Add this to a microfuge tube with enough PBS to equal 1 ml (1000 ul). Mix thoroughly.

Example: (from above)
0.1 ul x 20 = 2.0 ul/1 ml

3) Let wells with samples and standards dry overnight. As it slowly dries, protein will bind to the plastic and evenly coat the wells.
4) Make sure wells are completely dry before continuing.
5) Add 250 ul/well of freshly prepared 2% non-fat milk to wells and incubate on shaker for 15 min. Flip plate into sink to remove milk and blot (via inverting and hard taps) on an absorbent paper towel.
6) Add 50 ul/well of diluted MAb 32B11antibody and incubate on shaker for 1 hr. Flip plate to remove and blot with paper towel (via inverting and hard taps). Wash with PBST 3x, blotting between washes.
7) Add 50 ul/well of biotinylated IgM antibody, diluted in 1% BSA, and incubate on shaker for 1 hour. Flip plate to remove and blot with paper towel (via inverting and hard taps). Wash with PBST 3x, blotting between washes.
8) Add 50 ul/well of ExtrAvidin peroxidase***, diluted in 1% BSA, and incubate for 1 hour on shaker. Flip plate to remove and blot with paper towel. Wash with PBST 4x, blotting between washes.

9) If you are making the color developer* by hand and not from the kit, begin working on this about 20-30 min before the ExtrAvidin peroxidase incubation is completed. Start by checking and adjusting the pH of the citric acid (pH 4.0). Then, carefully weigh out the ABTS powder and dissolve it in dH_2O. About 5 min before the ExtrAvidin peroxidase incubation is completed, measure out the citric acid and add the ABTS solution. **DO NOT** add the hydrogen peroxide until after the PBST washings (right before use). If you are using the Bio-Rad horseradish peroxidase kit, **DO NOT** mix the solutions together until right before use (after the PBST washings). For either method, if you are doing more than one plate at a time, the color developer should be made for each plate individually.

10) Add 50 ul/well of color developer* (must be quick and accurate).

*Color developer

Note: Instead of mixing these solutions individually and because the ABTS may not always be fresh by the time of delivery, a kit may be purchased from Bio-Rad that is stabilized for a year.)

Just before use mix:

Citrate buffer	ABTS soln.	30% H2O2
10 ml	200 ul	10 ul
7.5 ml	150 ul	7.5 ul
5 ml	100 ul	5 ul
2.5 ml	50 ul	2.5 ul

Citrate buffer: For 100 ml use 1.05 g of citric acid (not sodium citrate) and adjust to pH 4.0 using 6 N and 2 N NaOH. **Check pH before each use.**

ABTS solution: 0.015 g 2,2'-azino-di-(3-ethylbenzthiaoline sulfonic acid)/1 ml H2O (Must be fresh = doesn't turn a strong green color in water)

Hydrogen peroxide: To test freshness: A 1:1000 dilution of 30% H2O@ in PBS should have an optical density of ~0.7 at 230 nm (see p. 150 of Goding, 1986)

11) After addition of color developer, incubate 15 minutes and read at 405 nm with plate reader. Determine mg/g concentration from OD values.

OD value range for the ELISA standard curve

Typical with peroxidase

Concentration (ug/well)	OD value
0	0
0.005	0.200-0.450
0.01	0.550-0.750
0.02	1.000-1.250
0.04	1.650-2.000

Typical with phosphatase

Concentration (ug/well)	OD value
0	0
0.005	0.200-0.400
0.01	0.600-0.750
0.02	1.700-2.000
0.04	2.700-3.000

Note: Because this curve has a slight quadratic shape, it is advised to dilute samples at the top of the curve in half and recheck them. It is also advised to rerun the sample at the bottom of the curve at 2x the concentration. This will give you ideal concentrations right in the middle of the curve.

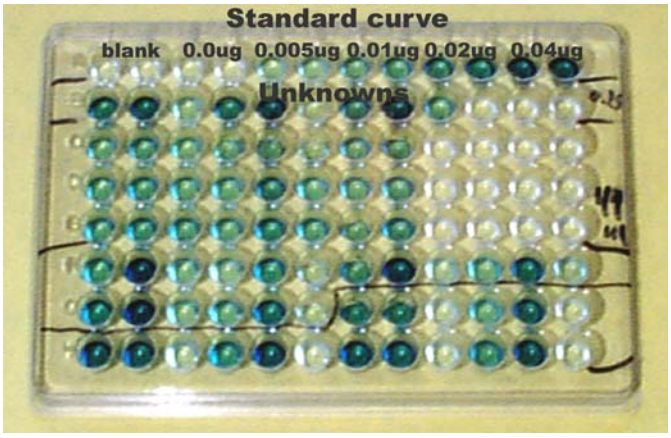

Figure 2. Example of a plate after addition of the color developer. Blanks are colorless while the intensity of green corresponds to concentration based on row A (standard curve).

Calculating mg/g concentration from OD values

1) After running the assay, you will have a value that gives you a protein concentration in a well of between 0-0.04 ug.
2) You take this number and divide it by the number of ul of sample that you placed in the well, giving you a ug/ul value.
3) You then multiply this number by the number of ul that you extracted (figured out by measuring the amount of extract you have using a graduated cylinder), resulting in an ug extracted value.
4) Finally, you divide this number by g weight that you extracted and you now have a ug/g value that can be converted in a mg/g value by dividing by 1000

Example:
OD reading (with peroxidase) = 1.150
ug/well concentration = 0.022 ug/well
ul of sample/well = 1 ul/well
amount of extract = 7650 ul (7.65 ml)
weight extracted = 1.0 g

0.022 ug/well ÷ 10 ul/well x 7650 ul ÷ 1.0 g = 1683 ug/g = 1.683 mg/g

Calculating percentage of immunoreactive protein (%IR)

By comparing this value with the total protein value obtained from the Bradford total protein assay, the amount of the protein that is immunoreactive can be determined, giving a % IR (immunoreactive).

Example: (continued from above)

% IR = (immunoreactive protein concentration ÷ total protein concentration) x 100
= (1.683 mg/g ÷ 1.9431 mg/g) x 100 = 86.61%

An Alternate Method for Color Development
ExtrAvidin Alkaline Phosphatase Method

Note: An alternate method is currently being applied in our lab where ExtrAvidin Alkaline Phosphatase is being used rather than the ExtrAvidin Peroxidase. This method has the potential for enhanced color development and is more stable because hydrogen peroxide is not being used.

1) Follow steps 1-5 as outlined in the methods procedure above.
2) Dilute the ExtrAvidin Alkaline Phosphatase in 1% BSA (3ul/6ml)* and add 50 ul of the diluted solution to each well.
3) Incubate for 1 hr. After incubation, flip plate to remove and blot with paper towel. Wash with PBST 3x, blotting between washes.
4) The fourth wash should be done with TBST not PBST, because PBST will react with the phosphatase enzyme. [TBST is Tris Buffered Saline (250 mM NaCl in 10 mM Tris (hydroxymethyl)aminomethane)) plus 0.2 ml/L of Tween 20 at pH 7.4.]
5) Dissolve one tablet of Sigma 104 Phosphatase Substrate (5 mg tablets) in 5 ml of DEA buffer (Mix 97 ml of diethanolamine buffer, 10%, with 1 L of 0.01% $MgCl_2$ solution, and adjust the pH to 9.8 with 1 N HCl. The solution must be kept sterile and stored covered at room temperature).
6) Add 50 ul to each well and incubate for 30 min. Read plate at 405 or 410 nm.
7) Calculate mg/g concentration from OD values.

* This is a recommended concentration. Chemicals obtained from different companies or with different lot numbers may need to be optimized for your conditions. If you are not getting the expected results, concentrations may need to be adjusted (see below).

Optimizing Concentrations

(Wright, 1994)

Because chemicals are obtained from different companies or have different lot numbers, the concentrations needed for optimal results with this highly sensitive assay may not match those suggested. If you are getting the expected results, concentrations may need to be adjusted. Besides the sample, there are four solutions used in the ELISA procedure (32B11 antibody against glomalin, anti-mouse IgM, ExtrAvidin Peroxidase or Phosphatase, and the color developer). You may need to adjust one or more of these solutions or the incubation time of the color developer. To check incubation time, read the plate every 2 to 5 min and optimize time for full development of standards and samples but not over development of blanks. To determine which solutions need to be optimized, you may use a checkerboard dilution procedure on a plate filled with one sample at one concentration. This checkerboard dilution allows you to examine two of these solutions within a plate by keeping two solutions constant and serially diluting the other two or more solutions – one horizontally and the other(s) vertically across the plate (see Table 1 below and section 6.4 in the reference for further details). You may also want to try the concentrations suggested by the manufacture or performing a serial dilution from some value that is 2 or more times the maximum to determine the optimum or minimal concentration.

Table 1. An example of the way to check for optimum concentration or to discover which chemical may not be performing correctly and needs to be reordered. On this grid representing a 96-well plate, 5 ul (or 0.02 ug) of a glomalin sample from a Baltimore soil was placed in each well. In the rows, the 32B11 antibody was serially diluted from four times the recommended concentration (4 ml in 2 ml PBS). In the first six columns, the ExtrAvidin Phosphatase was serially diluted from three times the concentration recommended in the procedure presented herein (9 ul in 6 ml 1% BSA in PBS). The remainder of the plate was covered with Scotch tape to use in another dilution series if necessary.

0.432	0.500	0.555	0.578	0.526	0.317						
0.679	0.978	1.356	1.659	1.153	0.330						
1.049	1.468	1.502	1.975	1.009	0.157						
1.429	1.519	1.624	1.725	1.036	0.149						
0.658	0.552	0.468	0.538	0.487	0.189						
0.468	0.579	0.456	0.339	0.285	0.090						
0.135	0.182	0.199	0.134	0.145	0.079						
0.075	0.129	0.157	0.055	0.039	0.008						

In this example, the optimum concentration was found in row 3, column 4, or at 1 ml for the 32B11 antibody and ca. 1 ul for ExtrAvidin Phosphatase. At the higher concentrations, the molecules may be self-aggregating and causing a reduction in values.

After optimizing the assay for your conditions, if unexpected results occur, one or more of the chemicals may need to be replaced. Use the checkerboard dilution procedure discussed above to determine which chemical needs to be replaced. You might also try replacing solvents such as the PBS used for the 32B11 antibody, the BSA for the anti-mouse IgM or ExtrAvidin, or the solutions used in the color developer (citric acid, hydrogen peroxide, or DEA).

Note: It is a good idea to make several plates full (all 96 wells) of the standard curve dried to the wells when you have a good sample that you are using as your standard, this will help to (1) solve problems with the antibodies or when your standards are getting too old,

and (2) can help you calibrate a new standard sample. Experimentation in your own lab using your best judgment should solve most of the problems that you may be having.

Changes in immunoreactivity

(Nichols, 2003; Nichols and Wright, 2004)

Glomalin is a complex biomolecule that has been recently identified. The exact composition of this molecule has yet to be determined. Therefore, prior to extraction, during extraction, and while analyzing, purifying and storing glomalin, certain changes in the conformation and folding pattern of glomalin may occur that would obscure the epitope for the antibody against glomalin (32B11) and reduce immunoreactivity. Although ELISA is useful in measuring glomalin specifically, it may be underestimating the concentration of glomalin, especially for glomalin extracted from soil. Some of the actions that could induce changes in immunoreactivity are:

1. Binding of iron, other cations, or organic matter to glomalin in soil or pots
2. Prolonged exposure to heat during the extraction process
3. Leaving samples in an aqueous citrate solution for more than a week or two by reactions with citrate and hydrophobic interactions
4. Growth of other microbes in the citrate solution
5. Exposure to pH extremes during the purification procedures
6. Freezing glomalin to obtain a freeze-dried sample

As more information is obtained about the structure of glomalin, ways to prevent reductions in immunoreactivity and improve the efficiency of extraction and quantification will be identified.